Research Article

JOURNAL OF HEPATOLOGY

Inhibiting poly ADP-ribosylation increases fatty acid oxidation and protects against fatty liver disease

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Background & Aims: To date, no pharmacological therapy has been approved for non-alcoholic fatty liver disease (NAFLD). The aim of the present study was to evaluate the therapeutic potential of poly ADP-ribose polymerase (PARP) inhibitors in mouse models of NAFLD.

Methods: As poly ADP-ribosylation (PARylation) of proteins by PARPs consumes nicotinamide adenine dinucleotide (NAD^+), we hypothesized that overactivation of PARPs drives NAD^+ depletion in NAFLD. Therefore, we assessed the effectiveness of PARP inhibition to replenish NAD^+ and activate NAD^+-dependent sirtuins, hence improving hepatic fatty acid oxidation. To do this, we examined the preventive and therapeutic benefits of the PARP inhibitor (PARPi), olaparib, in different models of NAFLD.

Results: The induction of NAFLD in C57BL/6J mice using a high-fat high-sucrose (HFHS)-diet increased PARylation of proteins by PARPs. As such, increased PARylation was associated with reduced NAD^+ levels and mitochondrial function and content, which was concurrent with elevated hepatic lipid content. HFHS diet supplemented with PARPi reversed NAFLD through repletion of NAD^+, increasing mitochondrial biogenesis and β-oxidation in liver. Furthermore, PARPi reduced reactive oxygen species, endoplasmic reticulum stress and fibrosis. The benefits of PARPi treatment were confirmed in mice fed with a methionine- and choline-deficient diet and in mice with lipopolysaccharide-induced hepatitis; PARP activation was attenuated and the development of hepatic injury was delayed in both models. Using $Sirt1^{hep-/-}$ mice, the beneficial effects of a PARPi-supplemented HFHS diet were found to be Sirt1-dependent.

Conclusions: Our study provides a novel and practical pharmacological approach for treating NAFLD, fueling optimism for potential clinical studies.

Lay summary: Non-alcoholic fatty liver disease (NAFLD) is now considered to be the most common liver disease in the Western world and has no approved pharmacological therapy. PARP inhibitors given as a treatment in two different mouse models of NAFLD confer a protection against its development. PARP inhibitors may therefore represent a novel and practical pharmacological approach for treating NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is now considered to be the most common liver disease in the Western world [1]. NAFLD is quickly becoming an important public health concern due to the rising incidence of obesity in both children and adults [2]. The disease spectrum for NAFLD ranges from simple fatty liver (steatosis), to non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma, and finally it may result in liver-related death [3,4]. Moreover, as the prevalence of NAFLD escalates, NASH-related cirrhosis and hepatocellular carcinoma will increasingly become a major health care problem and a primary indication for liver transplantation [5,6]. Currently, the principal treatment for NAFLD/NASH is lifestyle modification by diet and exercise [7,8]. However, pharmacological therapy is indispensable as obese patients with NAFLD often have difficulty in maintaining improved lifestyles. In this context, farnesoid X receptor or peroxisome proliferator-activated receptor-α activators are currently being tested in clinical trials, but they are not yet approved [9].

Keywords: Non-alcoholic fatty liver disease; Poly ADP-ribosylation; Sirtuin; PARP inhibitor; PARylation; NAD.

Received 20 April 2016; received in revised form 13 August 2016; accepted 31 August 2016; available online 20 September 2016

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In the early stages of NAFLD, liver-specific metabolic adaptations lead to increased liver fat load from the periphery [10], which may promote mitochondrial dysfunction upon chronic fat accumulation [11]. Studies performed in humans and rodents have demonstrated that enzymatic activities involved in oxidative phosphorylation (OXPHOS) are reduced in NAFLD [10,12,13]. The major consequence of reduced OXPHOS activity is a reduction in ATP generation [12]. Another aspect associated with mitochondrial dysfunction in NAFLD is an increase in reactive oxygen species (ROS) production. This phenomenon is often associated with elevated expression of several cytokines, promoting an inflammatory state, which has been shown to be deleterious in NAFLD [14,15]. ROS has also been reported to impair OXPHOS, thereby inhibiting β-oxidation and perpetuating the continued progression of NAFLD [16].

Several recent studies indicate the potential involvement of poly ADP-ribose polymerase (PARP) activity in promoting metabolic dysfunction [17–20]. Interestingly, various metabolic disorders have been associated with elevated oxidative stress and DNA damage, which can subsequently induce PARP activity [21]. The hyperactivation of PARPs can initiate the programmed cell death pathway causing both ATP and nicotinamide adenine dinucleotide (NAD^+) depletion [22], which reduces mitochondrial function and β-oxidation of fats [18,19,21,23]. Interestingly, this correlation was also found in the liver, as demonstrated by the negative correlation between *PARP1* and β-oxidation gene transcripts in normal human liver datasets [11].

Being an important substrate for PARP activity, NAD^+ is also a critical component for various metabolic reactions in cells [24]. For example, the sirtuin protein, Sirt1, regulates many metabolic pathways, in adaptation to nutritional status. When Sirt1 is active, during a state of energy deficit, it uses NAD^+ as a co-substrate for the deacetylation of proteins [25]. As NAD^+ is rate-limiting for Sirt1, the consumption of NAD^+ by PARPs can lead to reduced sirtuin activity [19,24,26]. We have previously shown that NAD^+ levels progressively decline with the development of NAFLD and postulated that this may be due to enhanced PARP activity and enhanced competition with Sirt1 for available NAD^+ [11]. Given the lower K_m for NAD^+ by PARP1 compared to Sirt1 this is a very likely scenario [24]. Based on these observations, the inhibition of PARP activity may represent a promising approach to counteract the development of NAFLD by increasing NAD^+ levels and activating Sirt1-directed mitochondrial biogenesis and β-oxidation of fatty acids.

In this study, we demonstrate that a long-term high-fat high-sucrose (HFHS) diet can induce hepatic PARylation, via increased PARP activity, having a deleterious effect on NAD^+/Sirtuin-directed metabolism, resulting in the development of NAFLD. By treating mice with a PARP inhibitor (PARPi) in a preventive and therapeutic manner, we were able to arrest or reverse the development of NAFLD. Moreover, using a liver-specific *Sirt1* knockout mouse line ($Sirt1^{hep-/-}$), we demonstrated that the PARPi-mediated protection against NAFLD development was dependent on hepatic Sirt1 expression. This protective effect of PARP inhibition against NAFLD was also confirmed using a methionine- and choline-deficient (MCD) diet, as another mouse model of NAFLD. Finally, we showed the benefit of PARP inhibition against lipopolysaccharide (LPS)-induced acute hepatitis in mice.

Materials and methods

Animal experiments

All animal experiments were performed according to Swiss, South Korean and EU ethical guidelines and approved by the local animal experimentation committee of the Canton de Vaud under license #2868 and the Committee on the Ethics of Animal Experiments of Chungnam National University Graduate School of Medicine (CNUH-015-A0001). Detailed experimental protocols are provided in the Supplementary Materials and Methods.

Generation of $Sirt1^{hep-/-}$ mice

Generation of $Sirt1^{L2/L2}$ mice has been previously described [27]. Liver-specific *Sirt1* knockout mice were generated by breeding $Sirt1^{L2/L2}$ mice with mouse albumin (Alb)-Cre mice ($Albcre^{Tg/0}$) [28], both of which have been backcrossed to C57BL/6J mice for 10 generations. These mice lines were then further intercrossed to generate mutant $Albcre^{Tg/0}/Sirt1^{L2/L2}$ mice, which were termed $Sirt1^{hep-/-}$ mice. $Sirt1^{hep-/-}$ and $Sirt1^{L2/L2}$ mice were fed with HFHS pellets containing vehicle (DMSO) or olaparib (PARP inhibitor, GP0126, Glentham Life Sciences, 50 mg/kg/day) for 18 weeks.

Histology and liver function

Preparation of histological tissue sections, staining procedures for hematoxylin and eosin (H&E), Oil Red O, Sirius Red, cytochrome c oxidase activity, succinate dehydrogenase activity and CD45 are described in the Supplementary Materials and Methods. Mitochondrial function in fresh liver tissue was evaluated with Western blotting, high-resolution respirometry [29], and BN-PAGE analysis [30], as described in the Supplementary Materials and Methods. Evaluation of global PARylation in fresh liver tissue was performed as previously described [19].

Quantification of NAD^+ and adenosine triphosphate levels

NAD^+ was isolated using acidic then alkaline extraction methods. Tissue NAD^+ was analysed with mass spectrometry, as previously described [11,31]. Total adenosine triphosphate (ATP) content was measured by the CellTiter-Glo luminescent cell viability assays (Promega). Typically, luminescence was recorded with a Victor X4 plate reader (PerkinElmer, Waltham, MA), and values were normalized by the total protein concentration, determined using a Bradford assay.

Liver triglyceride, cholesterol, and lipid peroxidation measurements

Hepatic lipids were extracted as described previously [32]. Triglyceride (TG) and cholesterol contents in hepatic lipid fractions were quantified using enzymatic assays (Roche). The by-product of lipid peroxidation (LPO) and a marker of oxidative stress, 4-hydroxynonenal (HNE), was measured following the manufacturer's protocol of the OxiSelect HNE-His Adduct enzyme-linked immunosorbent assay Kit (Cell Biolabs Inc., San Diego, CA).

Bioinformatic analyses

All raw transcriptomic data are publicly available on Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) under the accession numbers GSE48452, GSE24031, GSE28619 and GSE50579 or on GeneNetwork (www.genenetwork.org). Heatmaps were built using GENE-E (The Broad institute, www.broadinstitute.org/cancer/software/GENE-E/) Principal component analysis, corrgram and correlation network were accomplished using functions and packages in R (www.r-project.org). The depth of the shading at the correlation matrices (corrgram) indicates the magnitude of the correlation (Pearson's r). Positive and negative correlations within the corrgram and correlation network are represented in blue and red, respectively. Only correlations with a $p < 1 \times 10^{-5}$ are displayed in the correlation network.

Statistical analysis

Statistical analysis was performed with Prism software (version 6.0; GraphPad Software Inc.). The significance of differences between two groups was determined by unpaired two-tailed Student *t* test. For comparison of multiple groups, we applied a one-way analysis of variance (ANOVA) with a post-hoc Bonferroni

test. Results are presented as mean ± standard error of the mean (SEM). A p value <0.05 was considered significant.

Results

PARylation and PARP expression positively correlate to NAFLD indicators in both humans and mice

To understand the nature of the role of PARPs in the development of human NAFLD, we first analysed human transcriptomes from a cohort of patients with NAFLD that included 40 samples (GSE48452) deposited in the NCBI GEO database [33]. These data demonstrate an increased expression of inflammatory and fibrogenic genes in liver tissues from patients with NASH, clearly indicating the pathology of NAFLD (Fig. 1A). In addition, the induction of *PARP* transcripts, including *PARP1*, the most abundant *PARP* transcript and active NAD^+ consuming enzyme in mice [23], occurs in the group of patients with NASH (Fig. 1A). Using a principal component analysis (PCA) we confirmed that the majority of *PARP* transcripts (in blue, Fig. 1B) have a strong positive correlation with fibrogenic genes (Fibro. PCA, Fig. 1B). Of note, anti-oxidant genes have negative correlations with *PARP* and fibrogenic genes (Fig. 1C). PARP, fibrogenic and inflammatory genes were also closely clustered as a gene network, suggesting that this strong relationship might promote the development of a fatty liver (Fig. 1D). Furthermore, *PARP* transcripts were increased in datasets from other liver diseases, including alcoholic liver disease (GSE28619) [34] and hepatocellular carcinoma (GSE50579) (Fig. 1E and F) [35].

In line with these findings, *Parp1* transcript expression was also elevated in a cohort of mice that were classified according to their level of NAFLD induction when challenged with a high-fat diet (HFD) (GSE24031, Supplementary Fig. 1) [36]. Mice classified as high-responders to a HFD (HFH), meaning that NAFLD was more severe in these mice, expressed higher transcript levels of the various *Parps*, including *Parp1*. Furthermore, we observed that whole liver extracts from mice fed 18 weeks of a HFHS diet

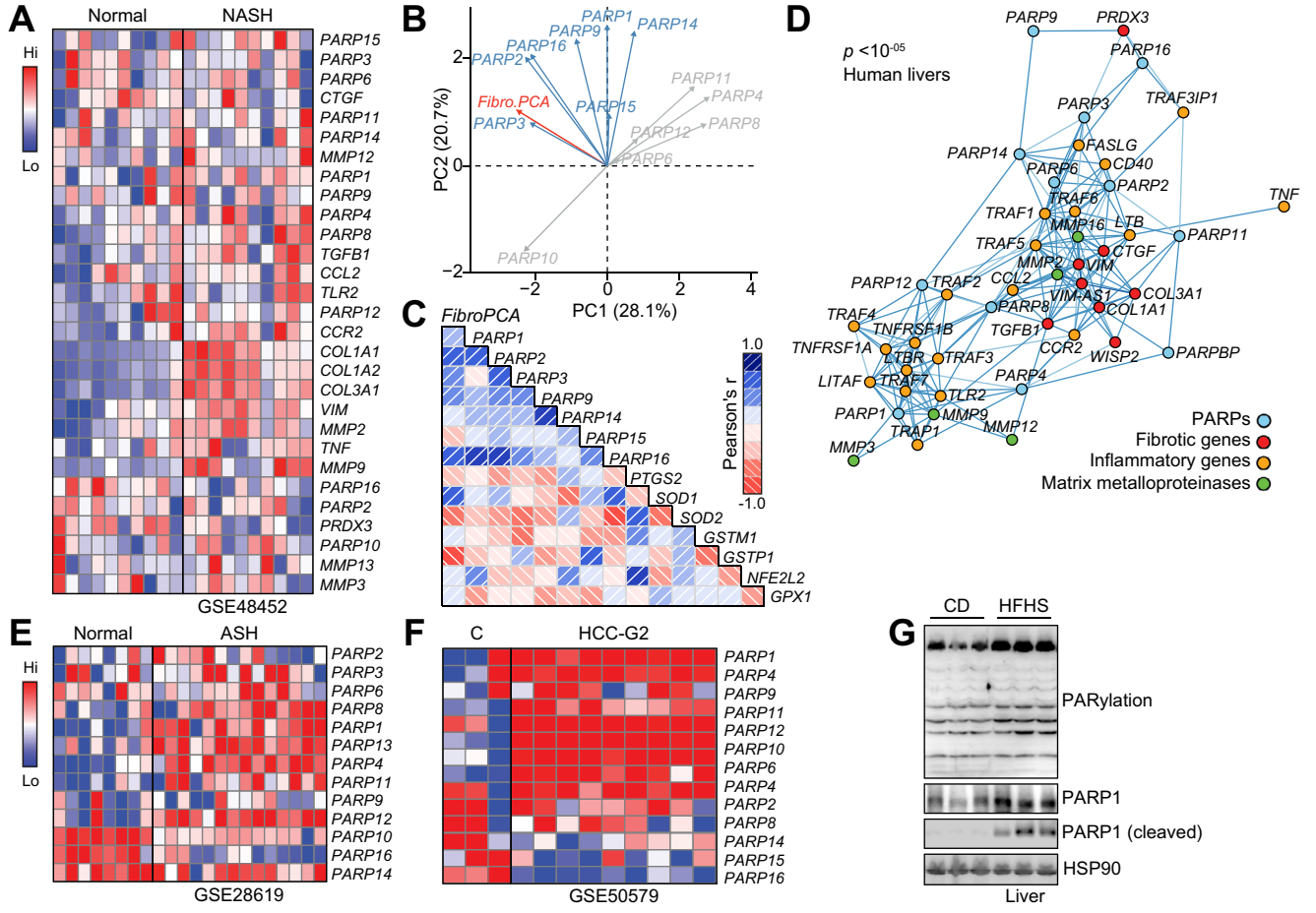

Fig. 1. Correlations between *PARP* transcripts and markers of inflammation or fibrosis in humans, along with PARylation and PARP expression in a mouse model of NAFLD. (A) Heatmap displaying the relative expression of *PARP*s and genes known to be inflammatory or fibrosis markers in normal control and NASH subjects (A; n = 10 per group; dataset GSE48452). (B) Principal component analysis (PCA) plot showing the relationship between *PARP*s and a fibrogenic PCA trait, generated from the fibrogenic genes in Fig. 1A and taken from the NASH subject datasets [33]. (C) Corrgram indicating Pearson's correlations amongst *PARP*s, the fibrogenic PCA trait, and selected anti-oxidant genes from the described NASH subjects [33]. (D) Correlation networks of *PARP*s, inflammatory and fibrosis-related genes in human liver samples derived from the GTEx project dataset (GSE45878, genenetwork.org). Only correlations with a $p < 1 \times 10^{-5}$ are displayed. (E–F) Heatmaps showing the relative expression of *PARP*s in patients with alcoholic steatohepatitis (E; normal control, n = 8; ASH, n = 14; dataset GSE28619), and in patients with hepatocellular carcinoma (F; control (C), n = 3; HCC-grade 2 (HCC-G2), n = 9; dataset GSE50579) (G) HFHS-induced global PARylation and PARP1 expression compared to livers derived from CD-fed mice.

(a Western style diet that leads to insulin resistance and adiposity [37]) expressed higher levels of PARP1 and global protein PARylation compared to chow diet (CD)-fed mice (Fig. 1G). All together, these data suggest that the induction of PARP expression and activity likely plays an important role in the development of NAFLD (and other liver diseases), leading us to hypothesize that reducing PARP activity, with clinically available PARP inhibitors, may help to attenuate the progression of NAFLD.

PARP inhibition improves fatty liver and systemic glucose homeostasis

To test our hypothesis, we treated mice either at the onset of a HFHS diet, in a preventive mode (starting at 7 weeks of age), or 9 weeks after the start of the diet, in a therapeutic mode (starting at 16 weeks of age), by supplementing the diet with the PARPi, olaparib (50 mg/kg/day). While food intake was not altered (Supplementary Fig. 2A), supplementing the HFHS diet with PARPi, in both preventive and therapeutic modes, reduced body, liver and epididymal fat weights compared to HFHS-fed mice in both preventive and therapeutic modes (Fig. 2A–C). In line with this finding, hepatic TG and total cholesterol levels were also reduced (Fig. 2D and E). These observations were concurrent to improved gross liver morphology and coloration in PARPi treated mice (Fig. 2F). Likewise, H&E and Oil Red O staining of liver tissue sections exhibited the expected histological features of HFHS diet-induced macrovesicular steatosis (Fig. 2G), which was attenuated by the administration of PARPi (Fig. 2G). PARPi treatment improved the histological score for steatosis, and showed a similar trend for the activity score, according to a blinded assessment by a certified pathologist (Supplementary Fig. 2B and C). In addition glucose homeostasis and insulin sensitivity was ameliorated in both preventive and therapeutic modes, as reflected by improved glucose excursion and reduced insulin release during an oral glucose tolerance test and the faster decline in glucose levels during an insulin tolerance test (Fig. 2H–J). PARPi-mediated protection from HFHS-induced hepatic injury was confirmed by reductions in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Supplementary Fig. 2D and E). No toxicity was observed upon PARPi treatment, as attested by normal kidney function, pancreatic and muscle enzymes (Supplementary Fig. 2F–H). Collectively, multiple major

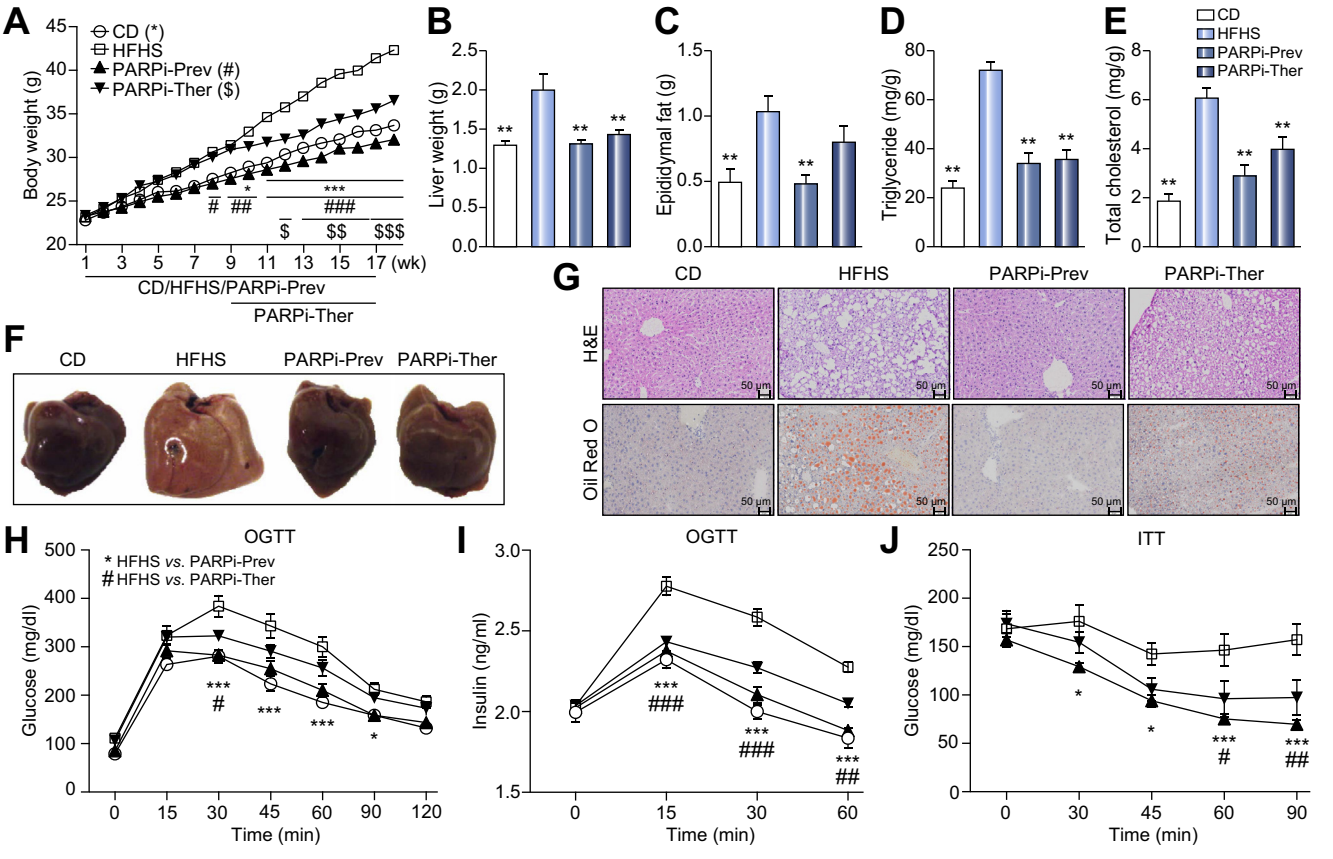

Fig. 2. PARP inhibitor (PARPi) treatment protects against and reverses NAFLD, while improving whole body glucose homeostasis. C57BL/6J mice were fed with CD, HFHS, HFHS + PARPi-Preventive (PARPi-Prev), or HFHS + PARPi-Therapeutic (PARPi-Ther). Phenotyping was performed after 5–18 weeks of treatment (PARPi dose: 50 mg/kg/day). The mice were sacrificed at 9 am after a 4 h fast. (A–E) PARPi reduced body weight (A; *, $^{\#}$, and $^{\$}$ indicates significant differences between HFHS and CD, HFHS and PAPRi-Prev, and HFHS and PARPi-Ther, respectively), liver weight (B), epididymal fat pad (C), liver triglyceride content (D), and cholesterol (E) in both preventive and therapeutic cohorts (n = 8–10). (F, G) Representative images of livers (F) and liver sections stained with H&E and Oil Red O (lipid content appears in red) (G), (n = 4–5). (H, I) PARPi therapy improved glucose tolerance (OGTT) (H) and plasma insulin levels (I) after an oral glucose tolerance test. (J) PARPi also improved insulin sensitivity during an insulin tolerance test (ITT), measured after 16 weeks of diet. (n = 8–10). *, $^{\#}$, $^{\$}$ p <0.05; **, $^{\#\#}$, $^{\$\$}$ p <0.001; ***, $^{\#\#\#}$, $^{\$\$\$}$ p <0.0001 compared to the HFHS cohort. Data are expressed as the mean ± SEM. One-way ANOVA with a post-hoc Bonferroni test was used for all statistical analyses. Male mice were used in these experiments.

clinical aspects of NAFLD were improved with PARP inhibition, indicating the multifaceted effects of PARP overactivation in NAFLD.

PARPi replenishes NAD$^+$ and improves mitochondrial function

PARP activity is associated with the depletion of cellular NAD$^+$, leading to a reduction in sirtuin activity and impairment of mitochondrial function [21]. First, we performed *in vitro* experiments using AML12 mouse hepatocytes and asked whether PARPi treatment is sufficient to increase cellular NAD$^+$ levels. Indeed, NAD$^+$ levels were induced in AML12 cells treated with PARPi for 24 h (Supplementary Fig. 3A). Meanwhile, PARPi also elevated mtDNA copy number (Supplementary Fig. 3B). Consequently, there was an induction of nuclear and mitochondrial DNA-encoded OXPHOS proteins in PARPi treated AML12 cells (Supplementary Fig. 3C).

Given that PARPi treatment can induce mitochondrial biogenesis *in vitro* by boosting intracellular NAD$^+$ (Supplementary Fig. 3A–C) and that a HFHS diet can induce the PARylation of proteins (Fig. 1G), we proposed that the PARPi might replenish liver NAD$^+$ levels by arresting the induction of PARylation during NAFLD. Indeed, HFHS-induced PARylation and NAD$^+$ depletion were both attenuated using either preventive or therapeutic PARPi administration (Fig. 3A and B). To assess whether the PARPi-induced restoration of NAD$^+$ levels impacted on mitochondrial biogenesis and function, we performed several additional analyses. First, we demonstrated that the ratio of mitochondrial DNA relative to nuclear DNA (mtDNA/nDNA ratio) in liver was higher in PARPi treated groups, indicating a higher mitochondrial content (Supplementary Fig. 3D). In line with an increased mtDNA copy number, we observed that PARPi treatment restored OXPHOS complexes and ATP levels in HFHS-fed livers (Fig. 3C and D), indicating an improvement in mitochondrial biogenesis and function. These observed beneficial effects on mitochondrial parameters were confirmed by increased cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) stains of liver sections, reflecting complex IV and complex II activity, respectively (Fig. 3E). PARPi induced *Cpt1*, *Mcad*, *Acox1* and *Cox5a* expression, while transcripts involved in lipogenesis, such as *Fasn*, *Acc* and *Scd1*, were reduced (Fig. 3F and Supplementary Fig. 3E). Taken together, the above findings demonstrate that PARPi therapy attenuates the consumption of NAD$^+$ by PARylation and improves the β-oxidation of fatty acids.

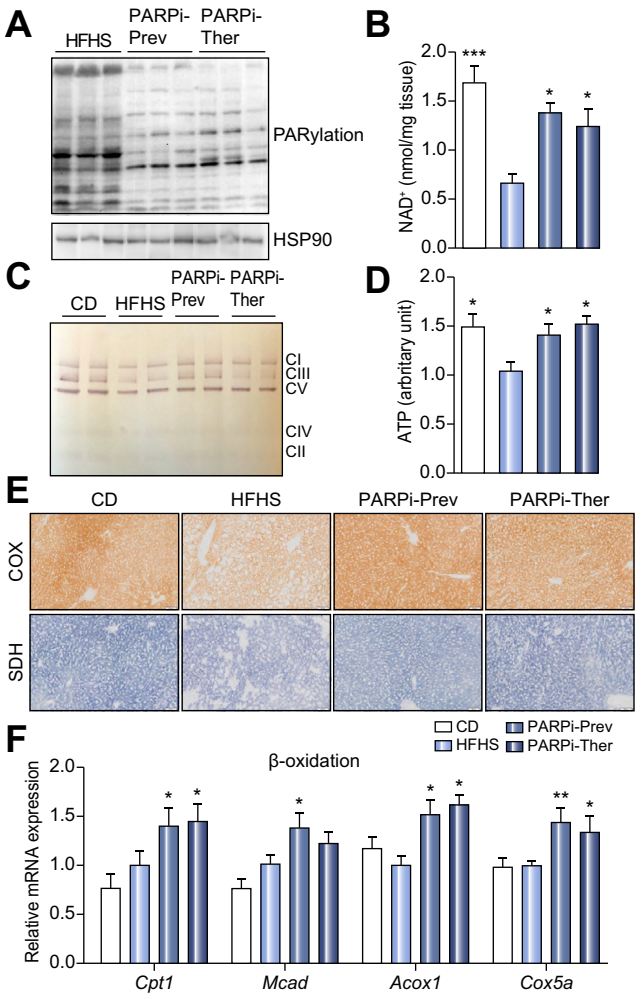

Fig. 3. PARPi induced mitochondrial biogenesis and function *in vivo*. (A) PARPi reduced global protein PARylation, (B) and replenished NAD$^+$ levels in liver tissue n = 6, (C) PARPi increased mitochondrial complexes as evidenced by blue native PAGE of isolated liver mitochondria. (D) PARPi increased ATP levels (n = 6). (E) Representative liver sections show improvement with COX and SDH activities in PARPi-treated groups compared to HFHS-fed mice. (F) PARPi induced expression of β-oxidation genes in liver tissue (n = 6). $^*p$ <0.05; $^{**}p$ <0.001; $^{***}p$ <0.0001. Data are expressed as the mean ± SEM. Two-tailed Student's *t* test or one-way ANOVA with a post-hoc Bonferroni test were used for statistical analyses. Male mice were used in these experiments.

PARP inhibition resolves hepatic endoplasmic reticulum stress and inflammation in HFHS-fed mice

Endoplasmic reticulum (ER) stress is critical to the initiation and integration of inflammatory pathways [38]. In human subjects with NAFLD, the activation of ER stress has been shown to correlate with histological severity [39]. Both the activating transcription factor 4 (ATF4), which is a central mediator of the ER stress pathway, and the CCAAT/enhancer-binding protein homologous protein (CHOP, also known as DDIT3 or GADD153), a transcription factor that is induced in response to a wide variety of cellular stresses, including ER stress, cell cycle arrest and apoptosis [40,41] contribute to hepatic inflammation [42,43]. As expected, HFHS diet effectively triggered the ER unfolded protein response (UPR^{er}) at both the transcript and protein levels in mice (Fig. 4A and B). Specifically, induction of the UPR^{er} signaling pathway, starting with ATF4, which transactivates both CHOP and the promoter of the binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78) [38], was shown to have higher protein and/or transcript expression in mice on a HFHS diet for 18 weeks. PARPi administration was associated with a decrease of these transcripts and protein markers of ER stress. This effect is in line with a previously described treatment using the NAD$^+$ precursor nicotinamide riboside (NR) [11].

PARPi therapy arrested the induction of inflammatory genes, such as *Il1*, *Il6*, *Ccl2*/*Mcp-1* and *Tnfa* in animals on a HFHS diet (Fig. 4C) [44]. These observations were in agreement with reduced plasma tumor necrosis factor alpha (TNFα) levels (Fig. 4D) and the infiltration of CD45-positive leukocytes into the liver (Fig. 4E). Furthermore, the preventive and therapeutic PARPi treatments similarly induced transcript levels of the anti-oxidant genes *Sod2*, *Cat*, *Gpx4* and *Prdx3*, compared to the

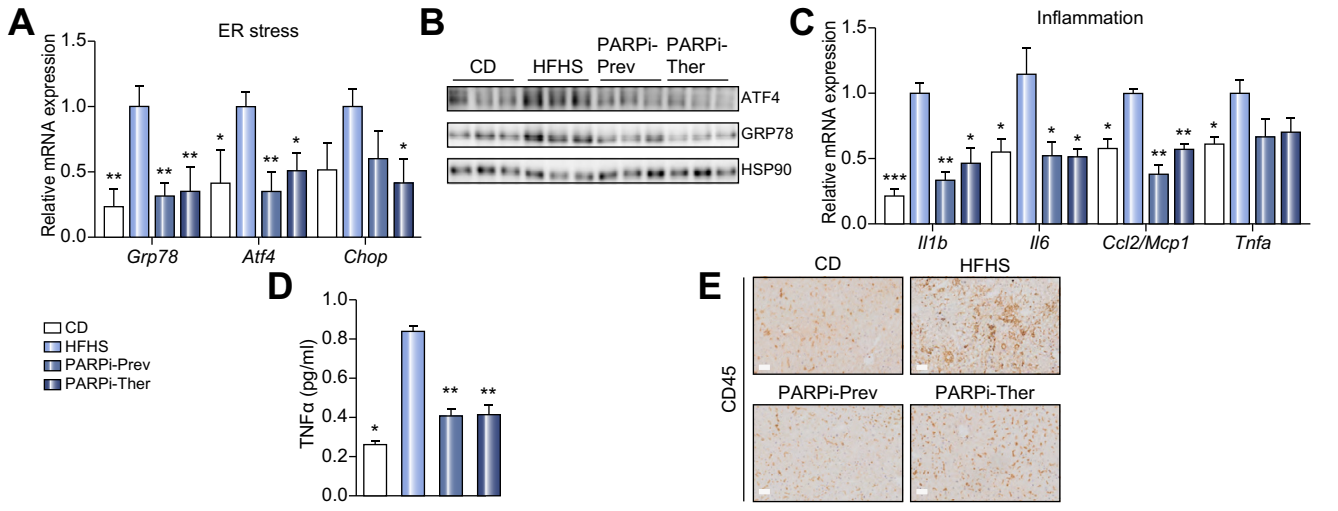

Fig. 4. PARPi therapy ameliorates ER stress and inflammation induced by a HFHS diet. (A, B) HFHS diet-induced transcripts involved in the UPR^{er} pathway, including *Atf4*, *Chop*, and *Grp78*, were reduced after PARPi treatments (A); this was, confirmed at the protein level for ATF4 and GRP78 (B). (C) PARPi reduced the expression of inflammatory markers in liver (n = 6). (D) Preventive and therapeutic PARPi treatments also attenuated HFHS diet-induced levels of plasma TNFα (n = 5). (E) Representative images of liver sections stained with CD45 antibody demonstrated reduced infiltration of leucocytes with PARPi. CD45 positive cells appear brown. (n = 4). $^{*}p$ <0.05; $^{**}p$ <0.001; $^{***}p$ <0.0001. Data are expressed as the mean ± SEM. One-way ANOVA with a post-hoc Bonferroni test was used for all statistical analyses. Male mice were used in these experiments.

reductions seen on a HFHS diet (Supplementary Fig. 3F). The resulting anti-oxidant protection in PARPi treated mice was validated by a decline in liver lipid peroxidation (Supplementary Fig. 3G), a surrogate marker for oxidative stress [45]. Altogether PARPi treatment conferred protection against the development of ER stress, inflammation, and ROS production, each being essential markers for the pathogenesis of NAFLD.

PARPi therapy prevents fatty liver disease induced by an MCD diet

To validate PARP inhibition as a robust treatment for NAFLD, the effect of PARPi was also examined in mice given an MCD diet. Feeding mice with an MCD diet is known to induce severe liver steatosis, inflammation, oxidative stress, and mitochondrial damage, without the classical systemic features of NAFLD such as insulin resistance [46]. At 10 weeks of age, male C57BL/6J mice were challenged with an MCD diet for 5 weeks. Similar to the effects seen in mice on a HFHS diet, MCD-fed mice treated with PARPi in a preventive manner exhibited reduced PARylation and increased hepatic NAD^+ levels (Fig. 5A and B).

Mice fed with an MCD diet for 5 weeks showed classical pathophysiological characteristics of NAFLD, including hepatic steatosis, inflammation and fibrosis. MCD diet increased AST and ALT levels compared to a control diet, while PARPi treatment reduced their levels (Fig. 5C and D). The protective effect of PARPi against MCD-induced liver damage was associated with increased oxygen consumption rates, driven by complex I (and trending with complex II) in the presence of ADP (Fig. 5E). This improvement of mitochondrial function by PARPi was complimented with an induction of *Acox1*, *Mcad*, *Cpt1*, *Cox5a* and *Cox2* transcript levels (Fig. 5F), with reduced Oil Red O staining of liver sections (Fig. 5G), and an improved steatosis, activity and fibrosis score (SAF score) for steatosis and activity (Supplementary Fig. 4A and B). This was matched with a reduction of ROS-induced damage, as revealed by diminished levels of lipid peroxidation in PARPi fed mice (Supplementary Fig. 4C).

Elevated hepatic expression of proinflammatory genes in MCD diet-fed mice was significantly reduced with PARPi intervention (Fig. 5F). These findings were confirmed by a reduction in CD45 positive stained cells in liver sections taken from PARPi treated mice (Fig. 5G). In agreement, PARPi lowered the expression of the fibrosis-related transcripts, *Col1a1*, *Col2a1* and *Col5a2*, in conjunction with a reduction in Sirius Red staining of liver sections (Fig. 5G and Supplementary Fig. 4D). This affirms previous observations that revealed protection by PARPi treatment against chemically induced hepatic fibrosis, in a context not linked to NAFLD and NAD^+ metabolism [47], demonstrating the potential multifaceted benefits offered by PARPi therapy. Similar to the HFHS experiment, we did not detect any signs of toxicity on key organs, including kidney, pancreas and muscle (Supplementary Fig. 4E–G). Altogether, the reduced lipid hepatic content, inflammation, lipid peroxidation and fibrosis in animals fed with an MCD diet demonstrates the broad protective role of PARPi therapy on the progression of NAFLD.

PARPi-induced mitochondrial biogenesis is Sirt1-dependent

We next tested the Sirt1-dependency of PARPi-induced mitochondrial biogenesis. In AML12 hepatocytes, the Sirt1 inhibitor, EX527, blocked the Sirt1-mediated deacetylation of FOXO1 seen with PARPi (Fig. 6A) *in vitro*. Along a similar line, we evaluated the potential Sirt1-dependency for PARPi action *ex vivo* using primary hepatocytes from *Sirt1*$^{L2/L2}$ mice, infected with an Ad-GFP (control) or Ad-Cre to induce a *Sirt1* loss of function. These experiments clearly demonstrate the Sirt1-dependency of the induction of the mitochondrial-encoded MTCO1 protein while the expression of the nuclear-encoded SDHA was not altered (Fig. 6B). The contribution of Sirt1 in mediating the effects of PARPi was next assessed *in vivo* using liver-specific *Sirt1*-deficient mice (*Sirt1*$^{hep-/-}$ mice) [27]. The Sirt1 dependency of PARPi action was demonstrated by the blunted response of liver and body weights in these *Sirt1*$^{hep-/-}$ mice, after 18 weeks

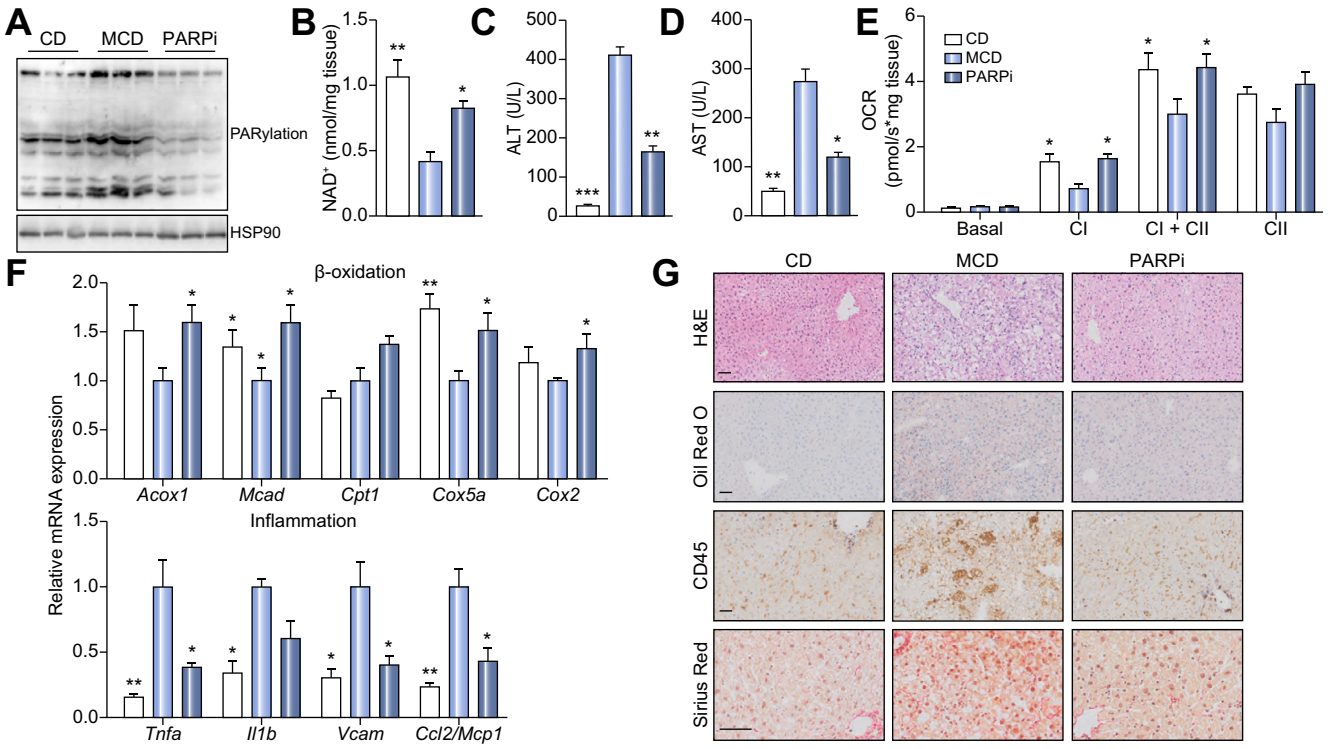

Fig. 5. Liver damage in MCD diet-induced NAFLD was reversed by NAD$^+$ repletion. C57BL/6J mice were fed with CD, MCD, or MCD+PARPi (PARPi, 50 mg/kg/day). The mice were sacrificed at 9 am after a 4-h fast. (A) PARPi reduces global protein PARylation and (B) recovers NAD$^+$ levels in liver tissue (n = 6). PARPi reduced circulating (C) ALT and (D) AST levels as indicators for liver damage (n = 6). (E) Increase in the OXPHOS complex I (CI)-coupled driven oxygen consumption rates (OCR), in the presence of glutamate, pyruvate, malate, and ADP and in CI and CII-coupled driven OCR, in the presence of succinate, occurred in mice treated with PARPi compared to mice under an MCD diet, while CII-coupled driven OCR, in the presence of rotenone were not significantly altered. (F) PARPi treatment increased the expression of genes involved in β-oxidation and reduced the expression of genes implicated in inflammation. (G) Representative images of liver sections stained with H&E, Oil Red O (lipid content appears red), CD45 antibody (CD45 positive appears brown), and Sirius Red (liver fibrosis represented by stained collagen), demonstrating reduced lipid content, inflammation and fibrosis, respectively, in mice treated with PARPi. $^*p$ <0.05; $^{**}p$ <0.001; $^{***}p$ <0.0001. Data are expressed as the mean ± SEM. One-way ANOVA with a post-hoc Bonferroni test was used for all statistical analysis. Male mice were used in these experiments.

of PARPi/HFHS diet (Supplementary Fig. 5A and B). These effects were complemented by the attenuation of the PARPi-mediated improvements in hepatic TG accumulation and fibrosis (Fig. 6C and Supplementary Fig. 5C), along with transcript levels in β-oxidation, including *Mcad*, *Cpt1 and Cox2*, and mitochondrial function, comprising of *Sirt1*, *Tfam*, *Tomm40*, *Atp5g1* and *Cytc*, in these *Sirt1*$^{hep-/-}$ animals (Fig. 6D). These findings suggest that Sirt1 is central to mediate the effects of PARPi on liver mitochondrial metabolism.

PARPi protects against LPS-induced acute hepatitis

To assess the potential of PARPi treatment in other liver diseases we evaluated its potential effect against LPS-induced acute hepatitis. At 8 weeks of age, male C57BL/6J mice were injected i.p. with LPS at 5 mg/kg of body weight. LPS injected mice exhibited severe hepatitis and systemic inflammation, leading to increased PARylation and NAD$^+$ depletion in their liver, all of which was alleviated by PARPi treatment (Fig. 7A and B). Plasma level of ALT was reduced in PARPi treated mice when compared to non-treated mice (Fig. 7C). Furthermore, PARPi treatment allowed for a significant reduction of LPS-induced hepatic proinflammatory genes *Il-1b* and *Il-6* (Fig. 7D). This was corroborated by a reduction in CD45-positive stained cells in liver sections taken from PARPi treated mice compared to non-treated controls (Fig. 7E). Altogether these observations showed that PARPi treatment might have a protective effect on other liver diseases, such as that of sepsis-induced acute hepatitis.

Discussion

Despite that NAFLD is now a worldwide burden on health, the exact pathophysiology and succession of events leading up to lipid accumulation, inflammation and fibrosis is not clearly delineated [14]. In particular, we recently described the progressive decline in NAD$^+$ homeostasis as a potential hallmark for NAFLD severity [11]. However, the cause for the decline in NAD$^+$ was, until now, not elucidated. As PARP proteins are major NAD$^+$ consumers and have been implicated in inflammatory responses, a common feature of NAFLD, we endeavored to profile the role of PARylation as a result of PARP activation in the development of NAFLD.

In a cohort of patients with NAFLD [33] we observed a negative correlation between transcript levels of several *PARP*s, including *PARP1*, with that of anti-oxidant genes, while fibrogenic genes were positively correlated. Furthermore, it was previously shown that *PARP* transcripts are negatively correlated to *NAMPT* and *NRK1*, two key enzymes involved in NAD$^+$ biosynthesis, in two independent normal human datasets and in the mouse

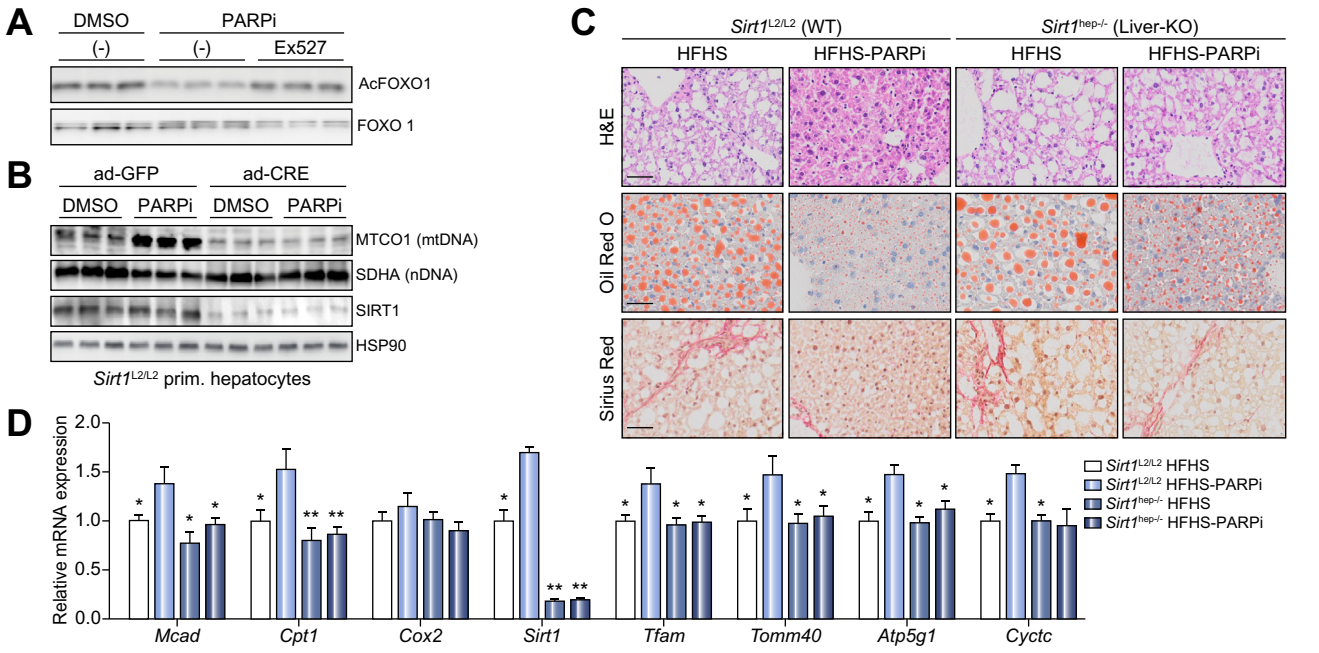

Fig. 6. PARPi-mediated liver benefits are mediated by *Sirt1* in the liver. (A) SIRT1 inhibition with the SIRT1 inhibitor EX527 (10 μM) in AML12 hepatocytes attenuated reductions in FOXO1 acetylation levels following PARPi treatment. (B) PARPi treatment of *Sirt1*$^{L2/L2}$ hepatocytes infected with an adenovirus expressing Cre attenuated the induction of the mitochondrial-encoded MTCO1 protein, while nuclear-encoded SDHA was not altered. (C) Representative images of *Sirt1*$^{L2/L2}$ and *Sirt1*$^{hep-/-}$ liver sections stained with H&E, Oil Red O and Sirius Red, demonstrating increased lipid content, and fibrosis in *Sirt1*$^{hep-/-}$ mice treated with PARPi on a HFHS diet, compared to *Sirt1*$^{L2/L2}$ mice. (D) Relative changes in transcript levels of genes associated with mitochondrial biogenesis and β-oxidation in mice of the indicated genotypes (n = 6). $^{*}p$ <0.05; $^{**}p$ <0.001; $^{***}p$ <0.0001. Data are expressed as the mean ± SEM. One-way ANOVA with a post-hoc Bonferroni test was used for all statistical analyses. Male mice were used in these experiments.

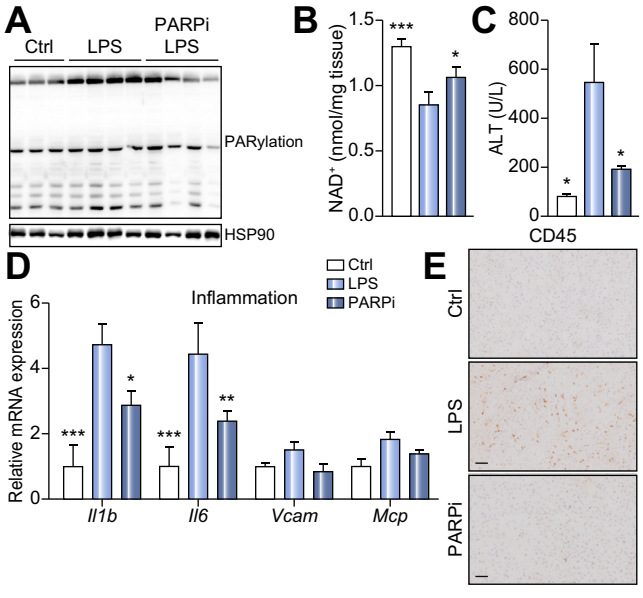

Fig. 7. PARPi treatment protects against LPS-induced acute hepatitis. (A) PARPi reduced global protein PARylation (B) and replenished NAD^{+} levels in liver tissue of LPS injected mice (n = 6). PARPi treatments also attenuated LPS-induced levels of plasma (C) ALT (n = 6). (D) PARPi reduced the expression of inflammatory genes in liver tissue (n = 6). (E) Representative images of liver sections stained with a CD45 antibody demonstrated reduced infiltration of leucocytes. CD45 positive cells appear brown (n = 4). $^{*}p$ <0.05; $^{**}p$ <0.001; $^{***}p$ <0.0001. Data are expressed as the mean ± SEM. Two-tailed Student's *t* test or one-way ANOVA with a post-hoc Bonferroni test were used for statistical analyses. Male mice were used in these experiments.

BXD genetic reference population [11]. Liver *Parp* transcript expression is therefore linked to markers of liver degeneration during the progression of NAFLD. These changes in human liver transcriptomes of patients with NAFLD were consistent with protein and enzymatic changes we observed in mouse models of NAFLD, as mice fed a HFHS diet exhibited increased PARylation and PARP1 expression matched with the depletion of liver NAD^{+} levels. Altogether, these findings demonstrate a conserved link between PARP1 expression, and the reduction of NAD^{+} levels during the progression of NAFLD. We therefore hypothesized that long-term PARP inhibition in HFHS-fed mice could recover NAD^{+} levels to activate sirtuins, which in turn would boost mitochondrial biogenesis and function, along with enhancing antioxidant levels and reducing inflammatory responses.

Treating mice in preventive or therapeutic modes with PARPi while on a HFHS diet recovered mitochondrial content and function leading to an increase in fatty acid β-oxidation and a reduction in hepatic lipid deposits. PARPi not only led to a marked reduction in the severity of diet-induced hepatic steatosis, but also improved glucose intolerance and systemic insulin resistance. Interestingly, we observed that mice given an MCD diet exhibited reduced β-oxidation gene expression, whereas mice under a HFHS diet showed elevations of the same gene-set. This suggests that the compensatory metabolic adaptations present in mice given a HFHS diet were lost in MCD-fed mice that expressed a more advanced stage of NAFLD [10].

The induction of mitochondrial biogenesis by PARPi was shown to be Sirt1-dependent by using a *Sirt1*$^{-/-}$ primary hepatocyte model. Similarly, feeding *Sirt1*$^{hep-/-}$ mice with a HFHS diet confirmed the SIRT1-dependent benefits of PARPi treatment.

The benefit of boosting NAD^+ to induce Sirt1-dependent mitochondrial biogenesis, as presently observed with PARPi, was previously shown by our group by supplementing a HFHS diet with the NAD^+ precursor NR [11,27,48]. However, the process of directly inhibiting PARPs, which are responsible for eliciting hepatic inflammation and fibrosis [49], may confer an advantage for the use of PARPi therapies over that of NAD^+ precursors. Altogether PARP inhibition may provide a multi-pronged therapy against NAFLD development by inhibiting the overactivation of PARPs, and therefore inflammation and fibrosis, while also safeguarding NAD^+ pools to activate Sirt1 and induce mitochondrial biogenesis and function. Along with reduced mitochondrial function, excessive oxidative stress was observed in both human and experimental models of hepatic steatosis and was suggested to be a key feature in the progression of NAFLD [50]. This characteristic increase in oxidative stress was recapitulated in the liver of HFHS- and MCD-diet-fed mice, which was attenuated with PARPi therapy, as revealed by the reduction in peroxidized unsaturated lipids. Our study hence shows that PARPi treatment improves mitochondrial function, reduces hepatic lipid accumulation, ER stress and ROS generation as previously reported in human NAFLD subjects following a bariatric surgery-induced weight loss, which was similarly characterized by a reduction of hepatic triglyceride content coincident to the attenuation of ER stress [51].

Both bioinformatics studies in humans, as well as, experimental work in two mouse models that mimic several features of human NAFLD [52], suggested that PARPs are involved in a wide range of alterations that predispose to the development of NAFLD. The manifestations of NAFLD, which can include abnormal hepatic metabolism, steatosis, inflammation and fibrosis, were all attenuated by PARPi treatment signifying the beneficial role of PARPi in the setting of NAFLD. We further explored whether PARPs were involved in other liver diseases. Our bioinformatics analysis suggested that increased PARP expression may also be associated with the development of ASH and HCC. We then extended the involvement of PARPs in multiple liver diseases by showing that PARP activation was also involved in sepsis-induced acute hepatitis and that PARP inhibition may likewise be beneficial as a treatment.

These findings emphasize the clinical relevance of PARP inhibition, not only in the field of oncology, but also as a promising therapeutic strategy for the treatment of NAFLD and potentially other liver diseases. Furthermore, NAD^+ balance was also shown to play a major role in acute alcohol-induced liver injury, suggesting that NAD^+ maintenance through PARP inhibition may be fundamental to preserve hepatic homeostasis [53]. Altogether, these observations have implications for the development of new approaches to prevent and treat this increasingly common cause of end-stage liver disease, PARP inhibitors are already FDA approved for the treatment of several cancers, leading to the potential rapid application of these pharmaceuticals for metabolic disorders such as NAFLD.

Financial support

K.G. is supported by a grant from the Geneva University Hospital, Switzerland and the Fondation Romande pour la Recherche sur le Diabète. K.J.M. is the receiver of a Heart and Stroke Foundation of Canada Award and is supported by the University of Ottawa. S.S. is supported by an SNSF-Ambizione grant. J.A. is the Nestlé Chair in Energy Metabolism. The research in the JA and KS laboratories is supported by École Polytechnique Fédérale de Lausanne, National Institute of Health (RO1AG043930), Krebs Schweiz/SwissCancerLeague (KFS-3082-02-2013, KFS-3444-08-2014), Systems X (51RTP0-151019), SNSF (31003A-124713, CRSII3_160798/1), SNSF (310030-143748) and Sinergia (CRSII3-160798). The research in Chungnam National University School of Medicine is supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT &Future Planning (No. NRF-2015R1A2A1A13000951).

Conflict of interest

JA is a founder and SAB member of Mitobridge, a company working on mitochondrial disease. The other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributors

KG initiated the project, designed and performed experiments, analysed the data and wrote the manuscript. DR, KJM contributed to design, performed experiments, analysed the data and wrote the paper. SS, HZ, AP, VL, EK, PJ, HSY, YKK, JTK, KSK, MS, SV, and LRB contributed to design and perform experiment and to analyse of the data. KS and JA designed the project and helped with the writing of the paper.

Acknowledgments

We thank S. Bichet, N. Moullan, and T. Clerc for technical help. The authors thank the members of the Auwerx and Schoonjans lab for discussions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.08.024.

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Author names in bold designate shared co-first authorship

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